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SOME PHARMACOLOGICAL AND CHEMOTHERAPEUTIC PROPERTIES OF NOTATIN

BY

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This paper includes descriptions of work carried out by colleagues in other member firms of the Therapeutic Research Corporation; such work is referred to under the name of the worker responsible; fuller reference to these colleagues is made in the acknowledgment at the end of the paper.

The nature and some of the properties of notatin have been described briefly by Coulthard *et al.* (1942) and Birkinshaw and Raistrick (1943) and in detail by Coulthard *et al.* (1945). The substances "penatin" (Kochalaty, 1942, 1943) and "penicillin B" (Van Bruggen *et al.*, 1943) are similar to, and probably identical with, notatin (Birkinshaw and Raistrick, 1943). Notatin is a flavoprotein enzyme catalysing the oxidation of glucose to gluconic acid by means of atmospheric oxygen, with the production of hydrogen peroxide. It exhibits high antibacterial activity *in vitro* in the presence of glucose, but is inactive in the absence of glucose or in the presence of catalase; the antibacterial activity therefore appears to be due to hydrogen peroxide formed during the oxidation of glucose.

The present paper is an account of some of the pharmacological properties of notatin, and of unsuccessful attempts to demonstrate an antibacterial action *in vivo*.

Acute toxicity and symptoms

The acute toxicity of the purest samples of notatin yet obtained is indicated by the following approximate median lethal doses:

		Mg. per kg. body weight
Mice.	Intraperitoneal	3
	Subcutaneous	4.5
	Intravenous	13
Rabbits.	Subcutaneous	7.5

The oral toxicity is low ; doses up to 300 mg. per kg. have no effect.

The effect of fatal intravenous doses has been observed in dogs, cats, rabbits, rats, and mice. In a cat or dog an intravenous dose of about 30 mg. per kg. rapidly produces severe cyanosis and anoxaemia. The haemoglobin of the blood is converted into a brown pigment which appears to be methaemoglobin ; at the same time the blood becomes more viscous and the clotting time is reduced. Death occurs in a matter of minutes, and cannot be averted by artificial respiration. At autopsy the lungs are grossly oedematous, and in many cases the liver shows marked degeneration. The general picture is similar in rabbits, rats, and mice, with minor differences. On the other hand, intravenous doses which are not immediately fatal appear to have no effect whatever ; this has been observed in cats, rabbits, and rats, with doses so large as to be only just sub-lethal.

The effect of fatal subcutaneous doses is different from that of intravenous doses ; a longer time elapses before death occurs (6 to 24 hours) and there is no observable blood-pigment change. The outstanding symptom in rabbits is a very marked rise in blood sugar, which is described below.

During the early stages of the production of notatin, as the samples obtained became successively purer, it was found that the toxicity became greater as the antibacterial activity *in vitro* became greater, indicating that the high toxicity of notatin is an inherent property of the active material and is not due to a separable impurity. This is confirmed by an observation by Miss Chapman that at least 90% of the toxicity disappeared when the antibacterial activity was destroyed by mild hydrolysis ; the stimulant action on smooth muscle and the antidiuretic action in rats were similarly decreased.

Effect on blood pigment

The effect of large intravenous doses on the blood pigment was investigated in the rat and the rabbit, and considerable differences were observed between the two species. In the rat, severe cyanosis was evident immediately the dose was injected, and the blood samples taken at any time after the injection showed the characteristic absorption spectrum of methaemoglobin. In the rabbit, although the blood gradually became darker after injection and was very considerably darker at the time of death, no methaemoglobin was detectable except in animals which had been anaesthetized with ether. The pigment causing darkening of the blood in unanaesthetized rabbits was not identified.

The change to methaemoglobin could be produced in rabbit blood *in vitro*, provided glucose was added and heparin was used as the anticoagulant. Methaemoglobin was not produced in the absence of added glucose, and not in any case if oxalate was present. Lysed washed red cells were affected in the same way as whole blood.

The fact that the only rabbits in which methaemoglobin formation was observed *in vivo* were etherized animals may be linked with the finding that a high glucose concentration is necessary *in vitro*, since the etherization of a rabbit raises the blood sugar.

Effect on carbohydrate metabolism

The effect of notatin on the reducing substances in blood was investigated in rabbits ; all estimations were done by the Hagedorn-Jensen method.

After subcutaneous injection of a dose in the region of the median lethal dose, either the blood sugar rose gradually, sometimes reaching very high levels, and the animal eventually died, or else there was no observable effect whatever, the blood sugar remaining normal and the animal appearing to be quite unaffected in any way. The larger doses produced the former response, while the smaller doses were without effect, but it was observed more than once that a dose which caused a rise in blood sugar and subsequent death in one animal would have no effect in another animal. The borderline dose was about 7.5 mg./kg. of the purest preparations.

In those animals in which a blood sugar rise occurred, death usually took place from 20 to 30 hours after injection, the blood sugar having risen to a maximum level of 300 to 500 mg./100 ml. ; one or two animals died later than 30 hours. Only two animals survived after a rise of blood sugar, and in both cases the maximum blood sugar level reached was about 250 mg./100 ml. ; in no case did death occur without a blood sugar rise preceding it.

In several animals insulin was injected subcutaneously when the blood sugar was at its maximum, and the latter then fell rapidly: unfortunately this phenomenon was difficult to observe, as the rabbit seemed unable to withstand the combined action of the two drugs, and invariably died soon after the injection of insulin. In a typical experiment the dose given was 7.5 mg./kg., and the blood sugar, initially 120 mg./100 ml., rose gradually until it was 300 mg./100 ml. 24 hours later: at this point 1.6 units of insulin were injected subcutaneously, and the blood sugar fell rapidly, reaching 72 mg./100 ml. 5 hours later; 15 hours after this it had risen again to 150 mg./100 ml., and death occurred a little later.

Glycosuria was observed in some of the animals with high blood sugars, and the liver glycogen and body temperature fell as the blood sugar rose, as shown by the experiment recorded in Table I.

TABLE I

EFFECT ON BLOOD SUGAR AND LIVER GLYCOGEN OF RABBITS

Treatment	Rabbit No.	Blood sugar in mg./100 ml.			Rectal temperature 9 hrs. after injection ° F.	Liver glycogen 9½ hrs. after injection Per cent.
		Initial	4½ hrs. after injection	9 hrs. after injection		
Notatin 15 mg./kg. subcutaneously	1	115	273	455	94·0	0·26
	2	125	255	450	98·4	1·72
	3	126	204	368	97·8	1·36
Controls	4	136	125	191	—	3·86
	5	137	124	137	102·8	5·40
	6	119	103	112	101·6	4·80

After intravenous injection of notatin into unanaesthetized rabbits, a small fall in blood sugar usually occurred: no considerable rise was ever observed.

Chronic toxicity

Rat growth tests carried out on five groups of five litter-mates of about 35 g. weight showed that daily subcutaneous doses up to the largest tolerated dose (0.8 mg./kg.), given for 14 days, had no effect on the growth rate.

A number of adult rabbits were given a daily subcutaneous dose of 2.5 mg./kg. for 14 days: there was no effect on the red cell count, haemoglobin or blood urea, but there was a marked granulocytosis which generally reached a maximum between the 5th and 8th days of dosage and then gradually returned to normal in spite of continuing daily doses. Rats given daily subcutaneous doses of 2 mg./kg. for 20 days showed a similar granulocytosis.

The toxicity to leucocytes was examined by Mr. Freeman, using a modification of the method of Thrower and Valentine (1943). Dilutions of notatin were prepared in normal citrated human blood and a staphylococcal suspension added. After incubation for 1 hour at 37° C., films were prepared and Gram-stained. The bacteria ingested into each of 25 phagocytes were counted, and a mean figure calculated. In the controls this was 21.4 cocci per phagocyte, and in notatin solution (1 in 200) 20.9 cocci per phagocyte. This concentration of notatin therefore did not inhibit phagocytosis.

Dr. Ungar has observed that in tissue cultures of chick embryo heart, notatin added to the nutrient plasma slightly inhibited the growth of fibroblasts in dilutions from 1 in 10,000 to 1 in 1,000, but some growth still occurred at a dilution of 1 in 250.

Local action

It was noticed throughout the experimental work that subcutaneous injections of notatin gave rise to oedematous swelling and pronounced tenderness at the site of injection. This was confirmed in *ad hoc* experiments on rabbits and rats; post-mortem examination showed widespread oedema, adhesions, and subcutaneous haemorrhage.

The action of notatin in uninfected wounds was observed in rats. The animals were anaesthetized and a small incision made in the skin of one leg: the underlying muscle was snipped with pointed scissors to a depth of 2 or 3 mm. and the skin incision was ligatured, all under sterile conditions. The opposite leg was treated similarly, but notatin was introduced before ligation. In most animals, approximately 0.5 mg. powdered notatin was introduced (about 2.5 mg. per kg. body weight), but in a few animals a solution was used (0.1 ml. of 0.2 per cent; equivalent to 1.0 mg. per kg. body weight). In three of the 12 rats used the foot distal to the notatin-treated wound became grossly swollen and oedematous 4 to 6 days later, the use of and sensation in the limb being temporarily lost. The swelling began to subside in 10 to 12 days and the limb eventually returned to normal in 2 of the 3 animals (the third was killed for examination). Of these animals, two had been treated with notatin powder and one with a solution. In the remaining rats little or no swelling occurred and no difference in degree of healing was apparent between the treated and control wounds. No systemic effects were observed in any of the animals.

The action of notatin in an infected wound was observed in a single rabbit. Wounds were made in each leg as before and both were heavily, and as far as possible equally, infected with *Staphylococcus aureus* (Mrs. Fox): one wound was then treated with 2 mg. powdered notatin. On the 4th day the notatin-treated leg was oedematous. On the 7th day the treated wound was deeply necrosed and very inflamed, while the untreated wound appeared to be healing well. On the 14th day both wounds were discharging pus, but the treated wound was clearly in a worse state than the untreated one. On the 24th day the

animal was killed and both wounds opened; the untreated one contained some pus, but appeared to be healing, while the treated wound contained much pus, showed considerable necrosis of skin and muscle, and was surrounded by swollen and inflamed tissue.

Inactivation of notatin in the body

Notatin loses its antibacterial action in the body. Large doses have been given to rabbits, both intravenously and subcutaneously, and samples of blood removed at varying intervals: in no case has any antibacterial activity been detectable in these samples when tested *in vitro*. Also the antibacterial activity *in vitro* is inhibited in the presence of whole blood, defibrinated blood or serum; this observation has been confirmed by Mr. Freeman. Heat treatment of serum destroys its power of inactivation.

Effect on diuresis

Two groups of eight male rats were given known volumes of water by stomach tube, after being deprived of food and water overnight. The animals in one group received a subcutaneous injection of notatin (0.3 mg./kg.) at the time of administration of the water, and the urine from both groups was collected for 30 hours. A week later the experiment was repeated with the same rats, the two groups being crossed over. The results were expressed as a mean percentage of the total water given which had been excreted as urine at various times after dosing, all sixteen animals being included in both treated and control groups. The results are given in Table II.

TABLE II

EFFECT ON DIURESIS IN RATS

Time after giving water and notatin	Percentage of total water excreted	
	Controls	Notatin treated
2 hours	51	40
4 hours	69	53
7 hours	72	54
22 hours	99	62
30 hours	107	66

Similar experiments were carried out with varying doses of notatin, and they indicated that single doses of 0.1 mg./kg. and upwards have a powerful anti-diuretic action, resulting in the retention in the body of a considerable portion of the water administered for a period of at least 30 hours.

Effect on smooth muscle

Notatin causes contraction of rabbit intestine and virgin guinea-pig uterus *in vitro*; concentrations of 1 in 40,000 down to 1 in 200,000 cause fairly powerful

contractions of the latter muscle; this observation has been confirmed by Miss Chapman. Dr. Wien has shown that notatin produces vasoconstriction in the perfused rabbit ear, in a concentration of 1 in 1,000.

Antibacterial activity in vivo

Tests of antibacterial activity *in vivo* were carried out in male mice of about 20 g. weight. A suspension of the organism was injected intraperitoneally, and this was followed immediately by an injection of notatin: in some cases this was the only dose of notatin, and in others it was the first of a series of doses at regular intervals.

Experiments were carried out with three organisms lethal to mice: *Streptococcus haemolyticus* (Richards), *Staphylococcus aureus* (C.N. 59), and a virulent strain of salmonella isolated from laboratory mice during an epidemic.

Notatin was administered in a variety of ways: subcutaneously, intravenously, and intraperitoneally; as a single dose, and as a series of doses at regular intervals; in doses up to 1.0 mg. per kg. body weight, the maximum tolerated dose; and in some cases with simultaneous injection of glucose. In most of the experiments penicillin or sulphonamides were used as positive control treatments.

The results obtained showed clearly that notatin had no therapeutic activity in these experiments. In view of the negative results, detailed protocols of the experiments are not given. Mr. Standfast also carried out an extensive series of experiments on mice infected with a strain of haemolytic streptococcus, with similar negative results.

Some experiments were also carried out with a strain of *Staphylococcus aureus* (Mrs. Fox) which is not lethal to mice; the usual criterion of therapeutic action—i.e., survival of animals which would otherwise have died—could not, therefore, be used. It was found that if a suitable infecting dose (of the order of 100 million organisms) was given by intraperitoneal injection, a number of small abscesses developed within a few days in the peritoneal cavity, and could be enumerated on autopsy, and an attempt was made to use the number of abscesses as a criterion of therapeutic effect.

A number of mice were given equal infecting doses, after which some groups were given subcutaneous injections of notatin or another drug, either as a single dose or as a series of doses spread over 24 hours, and other groups were left untreated to serve as controls. At a definite interval after injection (usually 72 hours) the mice were killed and autopsied, and the abscesses visible within the peritoneal cavity enumerated, no account being taken of variation in size. The counts varied from 0 to 9 abscesses per mouse.

The results were difficult to interpret. While the animals receiving the various drug treatments (notatin, penicillin, or sulphathiazole) generally showed lower abscess counts than the untreated controls, and the difference was often statistically significant, yet the degree of lowering was erratic and bore little relation to either the drug given or the amount. A given dose level of a given drug would produce a large and significant lowering of the count in one experiment, and have no effect in the next, while a larger or smaller dose of the same drug would behave in the opposite manner.

On the whole notatin caused a greater reduction of the abscess count than did penicillin, in contradistinction to the relative therapeutic activities in the other

in vivo tests (including those using *Staphylococcus aureus* C.N. 59) in all of which penicillin was effective while notatin was inactive. Certain aspects of the technique were investigated as possible sources of erratic variation in the counts, e.g., settling of the bacterial suspensions, and fatigue of the operator during the counting of the abscesses, but the erratic results could not be explained on these lines.

Some twenty experiments were performed in all; for reasons of space, the results cannot be given *in extenso,* but as the method has not been described previously, so far as we are aware, the protocol of one experiment is given in Table III. In this experiment both the mice treated with notatin and those treated with penicillin showed significantly lower counts than the controls.

TABLE III

EFFECT ON INTRAPERITONEAL ABSCESSES IN MICE

Organism. *Staphylococcus aureus* (Mrs. Fox).
Infection. 380 million organisms per mouse.
Treatment. Total dose of 0·1 mg. per kg. notatin, or 1,000 Oxford units per kg. penicillin, divided into two equal doses at 0 and 4 hours after injection.
Counted. 75 hours after infection.

Treatment	No. of Mice	Individual counts of intraperitoneal abscesses	Mean count
Controls	10	6, 6, 9, 6, 6, 5, 3, 6, 4, 6	5·7
Notatin	10	1, 0, 3, 2, 4, 4, 5, 4, 3, 4	3·0
Penicillin	10	5, 5, 5, 4, 5, 2, 4, 4, 2, 1	3·7

Discussion

It has been shown that notatin is highly toxic to animals. When given intravenously it interferes with the oxygen-carrying function of the blood by converting haemoglobin to methaemoglobin, and also causes gross oedema of the lungs. The two conditions proceed simultaneously, and death by asphyxia occurs rapidly. In the rat the blood pigment formed has been identified as methaemoglobin and this has also been found in the etherized rabbit; in the unanaesthetized rabbit the blood darkens, and loses the power of taking up oxygen, but the pigment formed does not appear to be methaemoglobin.

Notatin converts the haemoglobin of rabbit's blood to methaemoglobin *in vitro* only if excess glucose is added. It therefore seems probable that the formation of methaemoglobin is linked with the oxidation of glucose to gluconic acid by atmospheric oxygen, a process which is known to be catalysed by notatin; a reaction of the following kind may occur:

$$C_5H_{11}O_5CHO + Hb + O_2 \longrightarrow C_5H_{11}O_5COOH + HbO$$

Large subcutaneous doses of notatin cause death after several hours. There are no symptoms of asphyxia and the haemoglobin is unaffected, but the blood

sugar (at least in rabbits) rises considerably, at the expense of the liver glycogen. The toxicity is about three times greater when given subcutaneously than it is when given intravenously; together with the difference in symptoms, this leads to the conclusion that the mode of death is different in the two cases. The cause of death after subcutaneous injection is not clear, although it appears to be linked with the rise in blood sugar. It was not found possible to produce the symptoms of intravenous administration by giving notatin subcutaneously, however large the dose.

Sub-lethal subcutaneous doses given daily for a long period have no apparent systemic toxic effect. Subcutaneous injection or local application produce marked tissue damage, including oedema, haemorrhage, and necrosis. The only marked systemic effect of non-lethal doses which has been observed is a powerful anti-diuretic action; small subcutaneous doses cause retention of water in the body for 30 hours or more. The antidiuretic action and characteristic severe oedema produced by notatin suggest that it is a powerful capillary poison.

No definite evidence has been obtained of any antibacterial activity *in vivo*; mice were not protected from lethal doses of streptococci, staphylococci, or salmonella by notatin given either intravenously or subcutaneously. Since the antibacterial action of notatin *in vitro* in the presence of glucose is due to the hydrogen peroxide liberated, it is easy to understand its inactivity *in vivo*, as any hydrogen peroxide formed would be immediately destroyed by the catalase universally present in the blood and tissues.

The significance of the staphylococcal abscess experiments is doubtful. In many cases notatin reduced the number of intraperitoneal abscesses formed after injection of a non-lethal strain of staphylococcus in mice; other drugs of known antibacterial efficiency (penicillin and sulphathiazole) also produced this effect. There was, however, no relationship between the size of dose and degree of effect, and the relative efficiencies of the three drugs and of various doses of them ran quite contrary to their known relative efficiencies in normal *in vivo* experiments in which survival is the criterion. The abscess experiments cannot therefore be accepted as unequivocal evidence of antibacterial activity.

SUMMARY

1. The purest samples of notatin yet obtained have the following acute median lethal doses:

		Mg. per kg. body weight
Mice.	Intraperitoneal	3
	Subcutaneous	4.5
	Intravenous	13
Rabbits.	Subcutaneous	7.5

The oral toxicity is low; doses up to 300 mg. per kg. have no effect.

2. Fatal intravenous doses of notatin cause rapid death by anoxia due to pulmonary oedema and loss of the oxygen-carrying capacity of the blood; in some species severe methaemoglobinaemia occurs.

3. Fatal subcutaneous doses of notatin cause death several hours later: the cause of death is unknown, but death is associated with a progressive and marked rise in blood sugar (at least in the rabbit).

4. Local application of notatin causes marked tissue damage. Severe oedema, haemorrhage, and necrosis occur.

5. Single small subcutaneous doses of notatin cause retention of water in the body for periods of 30 hours or more. Notatin appears to act as a powerful capillary poison.

6. Notatin causes contraction of isolated smooth muscle, and produces vasoconstriction in the perfused rabbit ear.

7. Notatin does not exhibit antibacterial activity *in vitro* in the presence of blood or serum.

8. Notatin does not protect mice from lethal doses of streptococci, staphylococci, or salmonella.

9. Notatin sometimes reduces the number of intraperitoneal abscesses formed after intraperitoneal injection of a non-lethal strain of *Staphylococcus aureus* in mice, but it is doubtful whether this indicates any antibacterial action.

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